MYC overexpression imposes a nonimmunogenic phenotype on Epstein–Barr virus-infected B cells

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Lymphoblastoid cell lines, generated by immortalization of normal B cells by Epstein–Barr virus (EBV) *in vitro*, have strong antigen-presenting capacity, are sensitive to EBV-specific cytotoxic T cells, and are highly allostimulatory in mixed lymphocyte culture. By contrast, EBV-positive Burkitt lymphoma (BL) cells are poor antigen presenters, are not recognized by EBV-specific cytotoxic T cells, and are poorly allostimulatory, which raises the question of whether immunological pressure exerted during BL pathogenesis *in vivo* has selected for a 'nonimmunogenic' tumor phenotype. The present work addresses this question by examining the immunogenicity/antigenicity of cell lines, generated by conversion of a conditionally immortalized lymphoblastoid cell line to permanent growth independent of EBV-latent proteins by introduction of a constitutively active or tetracycline-regulated c-*myc* gene (A1 and P493–6 cells, respectively). Compared with its parental lymphoblastoid cell line, A1 cells showed many of the features of the nonimmunogenic BL phenotype, namely poor allostimulatory activity, poor antigen-presenting function associated with impaired proteasomal activity, down-regulation of peptide transporter, reduced HLA class I expression, and an inability to present endogenously expressed EBV-latent proteins to cytotoxic T cells. P493–6 cells, when grown in the presence of estrogen with the exogenous c-*myc* gene switched off, were strongly immunogenic. The cells had lost their immunogenic potential, however, when grown on a c-*myc*-driven proliferation program in the absence of estrogen. Deregulation of c-*myc*, a step central to the development of uncontrolled BL cell growth *in vivo*, can thus impose a nonimmunogenic phenotype on proliferating human B cells in the absence of any immune pressure.

Epstein–Barr virus (EBV), a B-lymphotropic human herpesvirus, infects primary resting human B lymphocytes *in vitro* and drives these cells into proliferating lymphoblastoid cell lines (LCLs) constitutively expressing six viral nuclear antigens (EBNAs) as well as three latent membrane proteins (LMPs) and two small nonpolyadenylated nuclear EBV-encoded RNAs (EBERs) (for review see refs. 1 and 2). Induction of the lymphoblastoid state is associated with growth in clumps, up-regulation of several cell activation markers including CD21, CD23, CD39, CD40, and CD71, increased expression of adhesion molecules like CD54 and CD58, up-regulation of the costimulatory molecules CD80 and CD86 (3), and acquisition of the ability to process and present antigen to T cells in the context of both HLA class I and HLA class II molecules (4, 5). When primary EBV infection leads to a virus-driven proliferation of LCL-like cells *in vivo*, as seen in patients with infectious mononucleosis, these cells elicit a strong EBV-specific $CD8^+$ cytotoxic T cell response, which efficiently controls the expansion (6). However, in a manner not yet understood, a few infected cells escape killing and gain access to the B cell memory compartment, the site where infected cells can persist lifelong (7). In T cell immunocompromised individuals, reactivation of EBV infection can lead to the uncontrolled outgrowth of EBV-positive immunoblastic lymphomas (posttransplant lymphoproliferative disease) closely resembling LCL cells; these tumors retain good antigen-presenting function and are susceptible to a restoration of T cell control (8).

By contrast, Burkitt lymphoma (BL) is a highly malignant B cell tumor that occurs with high frequency in tropical areas of Africa and New Guinea (so-called endemic BL) where it is consistently EBV-associated, and with lower frequency all over the world (sporadic BL) where its EBV association is less strong (for review, see ref. 9). Regardless of its geographical origin, the tumor invariably carries a chromosomal translocation involving the c-*myc* gene on chromosome 8 and one of the Ig gene heavy- or light-chain loci on chromosomes 14, 2, or 22, respectively (10, 11). EBV-positive BL and derived cell lines differ dramatically from LCLs in their cellular phenotype, growth behavior, and immunogenicity, as well as in their pattern of viral genome expression. Thus only one of the EBV-latent proteins, EBNA1, is expressed. EBNA2 and LMP1, the principal effectors that mediate B cell activation and play a crucial role in the process of B cell immortalization *in vitro*, are absent. As a result, EBV-positive BL cells do not express the activation markers, adhesion molecules, and costimulatory molecules typical of LCL (12, 13), and they grow as single-cell suspensions rather than in clumps. In addition, BL cells are deficient in their ability to present endogenously expressed proteins to $CD8^+$ T cells by way of the HLA class I pathway (14). Thus, certain components of that pathway, in particular the IFN-γ-inducible components of the proteasome and the peptide transporters TAP1 and TAP2, are down-regulated (15, 16). Furthermore, surface expression of some HLA class I alleles (e.g., A11) is markedly reduced, and BL cells show a very poor allostimulatory capacity in mixed lymphocyte cultures (17). In addition, the only EBV protein expressed in BL cells, namely EBNA1, is protected from degradation by the proteasome and thus not presented through the HLA class I-processing pathway (18, 19).

It has remained an open question whether the nonimmunogenicity of BL cells is primarily a consequence of immune T cell selection during tumor evolution *in vivo* or is a direct result of the genetic changes associated with malignant transformation. To address this question we have developed an *in vitro* model system that recapitulates some of the important features of BL pathogenesis. As a first step, we have constructed a conditional LCL (EREB2–5) by using a recombinant EBV expressing EBNA2 as

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Abbreviations: BL, Burkitt lymphoma; CTL, cytotoxic T cell; EBNA, Epstein–Barr virus nuclear antigen; EBV, Epstein–Barr virus; EBER, EBV-encoded RNA; LCL, lymphoblastoid cell line; LMP, latent membrane protein; PBMC, peripheral blood mononuclear cells.

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a hormone-regulatable EBNA2-estrogen receptor fusion protein (20). These LCL cells proliferate in the presence of estrogen and stop proliferating when estrogen is withdrawn from the medium. As a second step, we have introduced into the conditional EREB2–5 cells a constitutively active or a tetracycline-regulated c-*myc* gene to generate cell lines that can proliferate in the absence of estrogen (21, 22). Cell lines driven into proliferation by high c-*myc* expression in the absence of functional EBNA2 have adopted a phenotype and growth behavior very similar to that of BL cells and preferentially use another protein degradation pathway than the parental LCL cells (21, 23, 24). Here, we show that the cells proliferating on a c-*myc*-driven program have lost their ability to process and present antigens by the HLA class I pathway, implying that the nonimmunogenic phenotype of the Burkitt tumor is a direct consequence of the c-*myc* deregulation that is crucial to the process of malignant transformation.

Materials and Methods

Primary Cells and Cell Lines. Peripheral blood mononuclear cells (PBMC) were prepared from whole blood or buffy coats from healthy donors by Ficoll gradient centrifugation. Heparin was added as an anticoagulant. HLA-typed EBV-positive and EBV-negative donors were used. HLA typing was performed with serological methods for class I and with oligonucleotide-based methods for class II.

Cell lines EREB2–5 (20), A1 (21), and P493–6 (22) were established as described. With the exception of EBNA2, which is of EBV type I origin, all other EBV-derived antigens in these cell lines are of P3HR1 origin and therefore of EBV type II. These cell lines express the following HLA molecules: A11,28; B7,49; Cw7; DRw6,7; DQw1,2. All cells were cultured in RPMI medium 1640 supplemented with 10% FCS, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (complete medium). EREB2–5 cells were cultured in the presence of 1 μM estrogen. A1 and P493–6 cells were cultured continuously in the absence of estrogen and tetracycline. P493–6 cells that have been used as stimulatory cells were maintained under these conditions (EBNA2 *off*, exogenous Myc *on*) or cultured for 4 days in the presence of 1 μM estrogen (EBNA2 *on*, Myc *on;* "+/+"), in the presence of 1 μg/ml tetracycline (EBNA2 *off*, Myc *off*; "−/−"), or in the presence of estrogen and tetracycline (EBNA2 *on*, Myc *off*).

Cytotoxic T cell (CTL) clones IM73.1 and CM41 were established as described (25). CTL clone IM73.1 recognizes the peptide RPPIFIRRL (amino acids 379–387 of EBNA3A) in combination with HLA-B7. CTL clone CM41 recognizes peptide IVTDFSVIK (EBNA3B amino acids 416–424) in combination with HLA-A11.

Recombinant Vaccinia Viruses. Recombinant vaccinia viruses expressing EBNA3A, EBNA3B, and LMP2 were generated as described (26).

Antibodies. The following antibodies were purchased from Dianova, Hamburg, Germany: BB1 (α-CD80), W6/32 (α-HLA-ABC). Antibody IT2.2 (α-CD86) was purchased from PharMingen. Antibodies L243 (α-HLA-DR), B7/21 (α-HLA-DP), and TÜ 39 (α-HLA-DR/DP/DQ) were purchased from Becton Dickinson. All antibodies are of mouse origin. The following allele-specific monoclonal antibodies were used: AUF 5.13 (HLA A3, A11) (27), HB164 (HLA A11, A24) (28), and HB 56 (HLA B7) (29). Fluorescein isothiocyanate-conjugated goat-anti-mouse-Ig was purchased from Dianova.

Immunofluorescence Analysis. Fluorescence-activated cell sorter analysis was performed as reported (21). Samples were analyzed with a FACScan (Becton Dickinson) with CellQuest analysis software. For each sample a minimum of 10,000 cells were analyzed.

Assessment of Allogeneic PBMC Stimulation. Allogeneic PBMC stimulation was measured in a standard mixed lymphocyte culture. Mitomycin C-treated or γ-irradiated (20 Gy) stimulatory cells were incubated with allogeneic PBMC in microtiter plates in complete medium at a ratio of 1:1 (10^4 cells per well) for 4–8 days. For the determination of cell proliferation the cells were pulsed for the last 24 h with [^{3}H]thymidine. Incorporation of [^{3}H]thymidine into DNA was determined with liquid scintillation counting (30). Stimulation indices were calculated as stimulation index = $[(R + S) - S]/R$, where R is the proliferation of allogeneic PBMC in the presence of medium alone, S is the background proliferation of irradiated or mitomycin C-treated stimulatory cells, and $(R + S)$ is the proliferation of allogeneic PBMC in the presence of stimulatory cells. For each combination of PBMC and stimulatory cells, between 12 and 96 wells were prepared and data are expressed as mean stimulation index and SD, or as counts per minute after subtraction of background proliferation of stimulatory cells and PBMCs in medium alone.

Chromium-Release Assay. Cytotoxicity of cloned CTL was assessed by using the standard ^{51}Cr-release assay essentially as described (26). CTL were used at an effector-to-target ratio of 5:1. Percent specific lysis was calculated as $[(E - S)/(T - S)] \times 100$, where E is the experimental ^{51}Cr-release, S is the spontaneous release in the presence of medium, and T is the release in the presence of 0.1% Triton X-100. For vaccinia infection of target cells, cells were infected over night with recombinant vaccinia viruses. For peptide-pulsed target cells, cells were pulsed with relevant peptides or an equivalent dilution of dimethyl sulfoxide (negative control) at a concentration of 2 μg/ml for 2 h and then washed before adding to the microtiter plate.

Western Blot. Western blotting was performed as described (21).

Isoelectric Focusing of HLA Antigens. Isoelectric focusing was performed as described (31).

Results

A1 Cells Have Lost Their Ability to Stimulate Allogeneic T Cells in a Mixed Lymphocyte Reaction. A1 is a human B cell line that has been established by stable transfection of conditionally EBV-immortalized EREB2–5 cells with a c-*myc*/Igκ expression plasmid. In hormone-deprived medium, proliferation of A1 cells is driven by the c-*myc* gene in the absence of functional EBNA2, LMP1 (21), and LMP2 (G.W.B., unpublished observation). EBNA1 is expressed and is required for maintaining replication of the c-*myc*/Igκ expression plasmid. Because EBNA3A, -B, and -C are EBNA2 targets, they are likely not to be expressed, but final evidence for this absence of expression is missing because of lack of reagents for EBV type II EBNA3 proteins. We have shown that c-*myc*-transfected EREB cells (including A1) grow in single cell suspension and have down-regulated activation markers, adhesion molecules, and the costimulatory molecules CD80 and CD86 (21, 23, 32). HLA class I expression is reduced in A1 as compared with EREB2–5 cells by a factor of three as revealed by staining with the framework antibody W6/32 (Table 1). Likewise, HLA class II is also strongly down-regulated on A1 cells as compared with EREB2–5 cells, DR being affected more strongly then DP (Table 1). In line with the down-regulation of adhesion, costimulatory, and HLA class II molecules, A1 cells showed a significantly lower stimulatory capacity in allogeneic mixed lymphocyte reaction when compared with the parental EREB2–5 cell line. Fig. 1 shows the results of experiments assaying the proliferation induced in PBMCs from allogeneic donors when stimulated *in vitro* with cells of the EREB2–5 LCL

Table 1. MHC class I and II expression on EREB2–5 cells and A1 cells

Antibody	HLA antigen	EREB2–5*	A1*	Ratio†
Control		3	5	
W6/32	A, B, C	527	188	2.8
HB164	A11, A24	185	22	8.4
AUF5.13	A3, A11	271	24	11.0
HB56	B7	497	60	8.2
TU39	DR, DP, DQ	964	30	32.1
L243	DR	433	53	8.2
B7/21	DP	92	21	4.3

*Data are presented as mean fluorescence intensity.
†Ratio between the mean fluorescence intensity of the EREB2–5 cell line and the mean fluorescence intensity of the A1 cells.

vs. the A1 cell line. The EREB2–5 LCL induced a much stronger proliferative response than A1 cells.

Cytotoxic T Cells Are Unable to Kill A1 Cells Expressing Relevant Target Antigens from a Recombinant Vaccinia Vector. To investigate the antigen-processing function of A1 cells, cells were infected with recombinant vaccinia viruses expressing individual EBV EBNA3A and EBNA3B antigens. The parental EREB2–5 cells served as controls. These recombinant vaccinia viruses were chosen because of the availability of EBV-specific cytotoxic T cell clones restricted through two of the HLA alleles of the EREB2–5 and A1 cell genotype. Clone CM41 is HLA-A11-restricted and recognizes the IVTDFSVIK epitope from EBNA3B, whereas clone IM73.1 is HLA-B7-restricted and recognizes the RPPIFIRRL epitope from EBNA3A. As is clear from Fig. 2*A*, each of these clones shows strong recognition of the EREB2–5 target cells expressing the relevant target antigen from a recombinant vaccinia vector. By contrast, little or no recognition of the A1 target cells occurs after infection with the same vaccinia vectors. This lack of recognition was not caused by a failure of the vaccinias to infect A1 cells, because immunofluorescence assays confirmed equal levels of EBV antigen expression in target cells (data not shown).

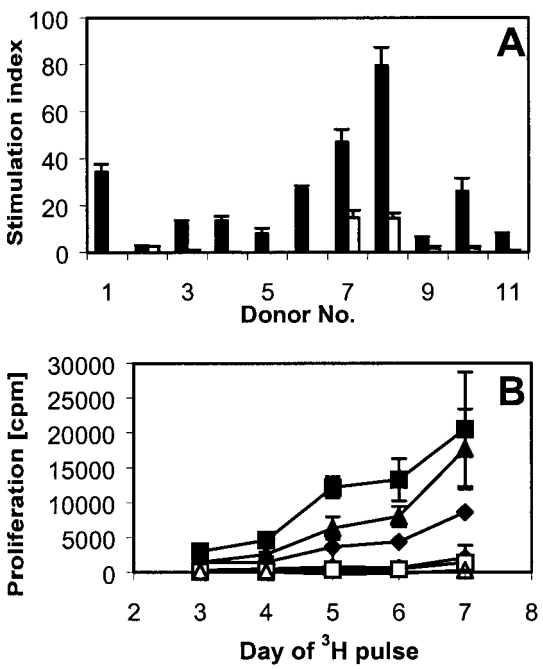

Fig. 1. Allostimulatory activity is abrogated in A1 cells proliferating in the absence of functional EBNA2. (*A*) Allostimulatory activity of A1 cells (open bars) and EREB2–5 cells (closed bars) was determined in a standard mixed lymphocyte culture with PBMC from different donors as responder cells. Stimulation indices were calculated as described in *Materials and Methods*. (*B*) Time course of allostimulation by EREB2–5 cells and A1 cells. PBMC from donors no. 9 (triangles), no. 10. (squares), and no. 11 (circles) were incubated for 8 days in the presence of EREB2–5 cells (closed symbols) or A1 cells (open symbols). Proliferation of PBMC was determined after a 24-h pulse with [^{3}H]thymidine at indicated times.

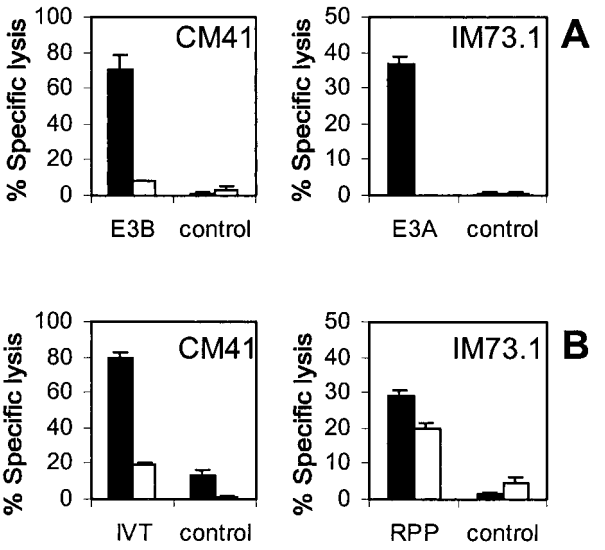

Fig. 2. Lysis by EBV-specific CTL is abrogated in A1 cells proliferating in the absence of functional EBNA2 and can be partially rescued by addition of the HLA-B7- but not the HLA-A11-restricted peptide. EREB2–5 cells (closed bars) proliferating in the presence of estrogen and A1 cells (open bars) proliferating in the absence of estrogen were used as target cells for CTL clones CM41 and IM73.1. (*A*) Target cells were infected with recombinant vaccinia viruses expressing EBNA3B (CM41), EBNA3A (IM73.1), or control vaccinia virus. (*B*) Target cells were pulsed with peptides IVTDFSVIK for clone CM41, RPPIFIRRL for clone IM73.1, and control peptides before incubation with the respective CTL.

A second series of assays then looked at T cell recognition after loading of the target cells with epitope peptides. As shown in Fig. 2*B*, EREB2–5 cells pulsed with the appropriate peptides were readily killed by the CTL clones. Remarkably, pulsing of A1 cells with the same peptides restored about 70% of killing by the B7-restricted T cell clone IM73.1, but only about 20% of killing by the A11-restricted CTL clone CM41, which suggested that HLA-A11 might be down-regulated in A1 cells in a more pronounced fashion than HLA-B7. As shown in Table 1, immunofluorescence analysis with allele-specific antibodies revealed down-regulation of both alleles on A1 cells compared with EREB2–5 to about the same degree (about 8-fold); however, HLA-B7 was expressed at slightly higher levels than HLA-A11. This finding is corroborated by studying the expression of the various HLA class I alleles by isoelectric focusing of immunoprecipitated HLA class I molecules from the A1 and EREB2–5 cells (Fig. 3). All those HLA class I alleles that could be identified unambiguously, namely A11, B7, and B49, were strongly down-regulated in A1 cells. We conclude that HLA-A11 is down-regulated in A1 cells below a critical threshold that is necessary to allow optimal CTL recognition by the addition of peptide, whereas HLA-B7 expression is sufficiently high to restore most of the cytotoxic activity upon preloading with the respective peptide. Two other HLA-A11-restricted CTL clones showed essentially the same pattern (data not shown).

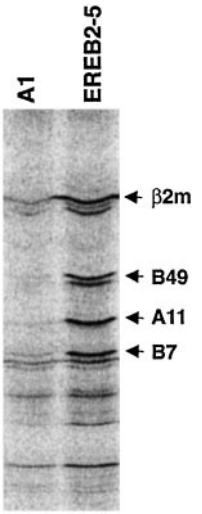

Fig. 3. Down-regulation of HLA class I alleles in A1 cells. Expression of HLA class I alleles was detected in A1 and EREB2–5 cells by isoelectric focusing.

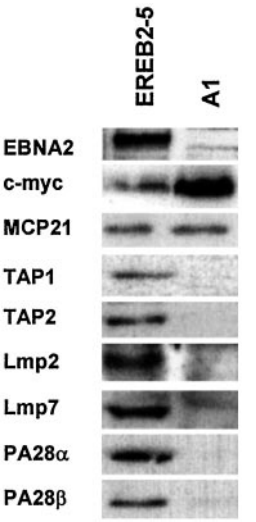

Fig. 4. Down-regulation of proteasome and peptide transporter components in A1 cells. A1 cells were cultured in the absence and EREB2–5 cells in the presence of estrogen. Expression of TAP1, TAP2, and proteasome components was studied by Western blotting with specific antibodies.

Note that, in these experiments, CTL clones CM41 and IM73.1 did not lyse the EREB2–5 control-target cells despite the fact that EREB2–5 cells naturally express all EBV-latent proteins. This finding was expected because EREB2–5 cells carry a type II EBV strain that does not possess the relevant type I-specific EBNA3A and EBNA3B epitope sequences recognized by these clones (33).

A1 Cells Are Defective in Processing and Presentation of HLA Class I-Restricted Antigens. From these assays, the inability of A11-restricted clones to recognize antigen-expressing A1 target cells may be explained by HLA-A11 down-regulation. However, the critical result, namely that the B7-restricted clone IM73.1 recognizes A1 cells when preloaded with the target peptide but not when overexpressing the target antigen, strongly reinforced the finding that A1 cells have a more fundamental impairment of the antigen-processing pathway (24). To look at the individual steps involved in this pathway, we compared the composition of the proteasomal components, the enzymatic proteasome activity and the expression pattern of the peptide transporters TAP1 and TAP2 in A1 and EREB2–5 cells. As shown in Fig. 4, the interferon-inducible components of the proteasome, namely lmp2, lmp7, PA28α, and PA28β, are significantly down-regulated in A1 cells as compared with EREB2–5 cells. In addition, both components of the peptide transporter complex TAP1 and TAP2 are undetectable in A1 cells. Consistent with a change in the proteasome composition, the enzymatic proteasome activity was also clearly decreased (16, 24). Therefore, the data strongly suggest that the inability of A1 cells to process and present the EBNA3A peptide as a HLA-B7:peptide complex reflects an impairment of the HLA class I-processing pathway at the level of epitope generation by the proteasome and/or at the level of peptide transport.

P493–6 Cells Exhibit the Immunogenicity of EREB Cells When Grown on an EBNA2-Driven Proliferation Program and the Nonimmunogenicity of A1 Cells When Proliferation Is Driven by c-*myc*. Even though A1 cells mimic almost all phenotypical and immunological features of BL cells, it is impossible to draw general conclusions from the analysis of a single cell line. To verify that the nonimmunogenicity of A1 cells is indeed caused by c-*myc* overexpression and the lack of EBNA2 and LMP1 expression, we have generated a second derivative of EREB cells in which c-*myc* expression can be regulated by tetracycline (22, 23). Depending on the culture conditions, this cell line can be grown on an EBV-driven or on a c-*myc*-driven proliferation program. As shown in Fig. 5*A*, P493–6 cells exhibited normal allostimulatory capacity for T cells when grown on an EBV-driven program and almost no activity when grown on a c-*myc*-driven proliferation program. Likewise, cells growing in the presence of estrogen and tetracycline were readily killed by the CTL clone CM41 upon infection with vaccinia virus expressing EBNA3B, whereas the same cells exhibited a severely reduced ability to be killed when grown on a c-*myc*-driven proliferation program (Fig. 5*B*). Contrary to A1 cells, addition of the IVT peptide rescued killing almost completely. This finding is consistent with the finding that in c-*myc*-driven P493–6 cells down-regulation of phenotypic markers including HLA class I (data not shown) is less pronounced than in A1 cells (23), but that the proteasomal enzymatic activity is markedly reduced (24). Reinforcing the similarities between A1 and P493–6 cells, we conclude that the nonimmunogenicity of A1 and P493–6 cells is a consequence of overexpression of c-*myc* concomitant with functional inactivation of EBNA2. If EBNA2 and Myc are expressed concomitantly, the LCL latency III phenotype is dominant (Fig. 5*A*), which was expected from the comparison of EBV-negative and B95–8 virus-infected BL cells (34) and from the comparison of individual clones of BL lines that have either maintained the latency I or acquired a latency III phenotype (35).

Discussion

Human tumors of B cell origin generally do express HLA class I as well as class II molecules and thus have an intrinsic capacity to induce an immune response, especially those tumors that are virus-associated and express viral antigens within tumor cells. Posttransplant lymphoproliferative disease and BL are both EBV-associated B cell malignancies and yet in pathogenetic terms are fundamentally different diseases. Posttransplant lymphoproliferative disease only arises in T cell-immunocompromised patients in whom cytotoxic T cell control over latent growth transforming infections of the B cell system has been lost; this tumor remains highly immunogenic and is sensitive to adoptive transfer of EBV-latent antigen-specific T cell preparations (8). EBV-positive BL on the other

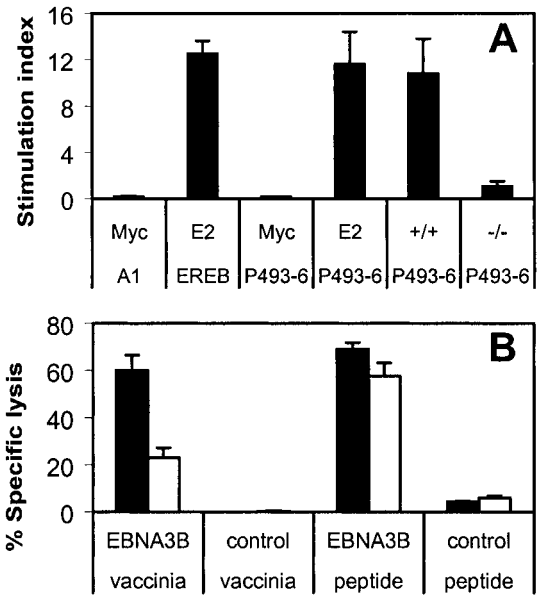

Fig. 5. Switching proliferation form a EBV-driven into a c-*myc*-driven program abolishes the immunostimulatory capacity of P493–6 cells. P493–6 cells were cultured in the absence or presence of estrogen and/or tetracycline. (*A*) Allostimulatory activity of P493–6, A1, and EREB2–5 cells was determined in mixed lymphocyte culture analysis with PBMC from an HLA-mismatched donor as responder cells. Stimulation indices were calculated as described in *Materials and Methods*. "Myc", "E2", "+/+" indicate expression of Myc and/or functional EBNA2, respectively; "−/−" no expression of Myc and EBNA2. (*B*) P493–6 cells were used as target cells for CTL clone CM41. Target cells were infected with recombinant vaccinia viruses expressing EBNA3B or control vaccinia virus or were pulsed with peptide IVTDFSVIK or irrelevant peptide before incubation with the CTL. Open bars, P493–6 cells cultured in the absence of estrogen and tetracycline; closed bars, P493–6 cells were cultured in the presence of estrogen and tetracycline.

hand arises in immunocompetent patients who still have evidence of EBV-specific immunity (36). This tumor shows multiple features of a nonimmunogenic phenotype. Besides the marked restriction of EBV antigen expression, BL cells express adhesion molecules and costimulatory molecules at a low level (12, 13), show selective down-regulation of some HLA class I alleles such as HLA-A11 (37), and exhibit a reduced capacity to process and present antigens in an HLA class I-restricted fashion (15, 16, 38). Consequently, the evolution of EBV-positive BL has been widely interpreted as an example of immunoselection *in vivo*. But, in contrast to other examples of immune selection where individual target antigens [e.g., MAGE in melanoma (39)] or HLA antigens [e.g., some haplotypes in cervical carcinoma (40)] are down-regulated without otherwise altering the tumor cell phenotype, BL cells seem to have acquired multiple redundant changes. This finding raises the possibility that these multiple changes in tumor cell immunogenicity may have been coordinately imposed by the process of neoplastic growth transformation itself rather than having been subject to an additional selective step imposed by immune pressure.

In this article we have addressed the question by re-creating certain crucial aspects of BL pathogenesis *in vitro* in the absence of any immune pressure. Starting from normal human cord blood lymphocytes we have generated a conditionally immortalized LCL whose proliferation can be turned off if estrogen is withdrawn from the medium (20). Proliferation of these LCL could be rendered independent of estrogen by stable transfection of a c-*myc* expression plasmid (21, 22). We have shown previously that the cells adopt the growth pattern and cell surface phenotype of BL cells. Here we present evidence that these cells do indeed display all features of the nonimmunogenic BL phenotype and as such are dramatically different from the parental EREB2–5 cells. Activation markers, adhesion and costimulatory molecules, are strongly down-regulated. In keeping with this change in the phenotype, the cells have a very poor allostimulatory capacity for T cells as compared with the parental LCL. HLA class I expression is strongly decreased. The remaining level of expression differs for various HLA class I alleles. For HLA-A11, expression is below the minimum threshold that allows killing in peptide sensitization assays, whereas residual HLA-B7 is above the threshold. However, when EBV antigens containing either HLA-A11- or HLA-B7-restricted T cell epitopes were highly expressed in the A1 cell line by using vaccinia vectors, no significant recognition either by A11-epitope-specific or B7-epitope-specific $CD8^+$ T cell clones occurred. This outcome clearly demonstrates that A1 cells, like BL-derived lines themselves, are fundamentally impaired in their ability to process endogenously expressed antigens by way of the HLA class I pathway. Subsequently, immunoblotting and functional studies showed that A1 cells do not express the inducible subunits of immune-proteasomes and have a decreased enzymatic activity as compared with the parental EREB2–5 cells (24). The peptide transporters TAP1 and TAP2 are also down-regulated. Most importantly, nonimmunogenicity is recapitulated in P493–6 cells that proliferate on a c-*myc* program, whereas the same cells proliferating on an EBV-induced program are highly immunogenic. We conclude that, in this *in vitro* system, B cells driven into proliferation through constitutive or conditional up-regulation of c-*myc* are immunologically silenced in the absence of any immune selection. Immunological silencing is therefore a by-product of more fundamental cellular changes caused by c-*myc* up-regulation on the one hand and down-regulation of EBNA2 and its target genes (including LMP1) on the other hand. These findings are entirely consistent with the observation that EBV-positive and EBV-negative BL show essentially the same nonimmunogenic phenotype.

It will be important to understand mechanistically how c-*myc* overexpression is able to impose a nonimmunogenic phenotype onto proliferating B cells. At least some of the genes involved in immune recognition will be, directly or indirectly, negatively regulated by c-*myc*. Several such examples have indeed been reported including HLA class I (41–43), CD58 (44), and CD23 and CD39 (45). By using the P493–6 cell system, some of the genes that are involved in the immunogenic phenotype have indeed been shown to be negatively regulated by c-*myc* and positively regulated by EBNA2 and its EBNA2 target genes (23, 46).

Besides a direct effect of the Myc protein on gene expression, an additional contribution of epigenetic silencing to the nonimmunogenicity of the cells has to be envisaged. Genes that are positively regulated by EBNA2 and its target genes but are neither positively nor negatively affected by Myc might be silenced epigenetically when cells proliferate on a Myc-driven program. We have observed that, upon prolonged proliferation of A1 cells in culture in the absence of estrogen, the EBNA2 gene becomes epigenetically silenced by methylation. Treatment with 5-azacytidine plus estrogen not only rescued EBNA2 expression in A1 cells but also immunogenicity (M.S.S. and A.P., unpublished observation). Again this fixation of the nonimmunogenic phenotype by epigenetic changes has occurred *in vitro* in the absence of immune selection. By using chromatin immunoprecipitation in the various conditions with either antibodies for Myc- or heterochromatin-specific proteins, the P493–6 cell line may represent an important tool to discriminate direct effects of

Myc on selected genes from those imposed by epigenetic silencing. Finally, it becomes important to know whether the effects of c-*myc* overexpression seen in our *in vitro* model can be reproduced *in vivo*. By using c-*myc*/Igκ (47) and c-*myc*/Igλ constructs (48), we have recently been able to generate transgenic mice that develop tumors histologically resembling BL (49). It will be interesting to look for evidence of immunological silencing by expressing foreign antigens in these tumor cells and, if silencing is observed, to see how this phenotype might be reversed by therapeutic regimens.

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